

# Commitment to Senescence: a Model for the Finite and Infinite Growth of Diploid and Transformed Human Fibroblasts in Culture

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As selection may be expected to act against senescent cells, the observed finite lifespan of human diploid fibroblast cultures suggests that cells become committed to senescence while still outwardly healthy. On this basis a mathematical model is developed for the growth and development of human diploid fibroblast cultures. The division of an uncommitted cell is assumed to produce committed daughter cells with a fixed probability,  $P$ , the "probability of commitment". An incubation period of  $M$  cell divisions must then elapse between commitment and the death of the resultant clone. This model is compatible with at least three possible ageing mechanisms.

The model predicts that an initially healthy population will double in size with each successive cell division (Stage 1 growth) until, after  $M$  divisions, the first deaths occur. For  $P > 0.5$  the population then becomes rapidly extinct. For  $P < 0.5$  a reduced but steady growth rate is adopted (Stage 2 growth). Unless  $M$  is sufficiently small, or the population size sufficiently large, the experimental routine of subculturing will lead to loss by dilution of the uncommitted cells and the population is then mortal. The final stage of growth (Stage 3) occurs as the last live cells become senescent and die.

The predicted growth is found to agree well with some experimental data for human fibroblasts. The model suggests a possible explanation for both the observed finite lifespan of human diploid fibroblast cultures and the apparent immortality of lines transformed by an oncogenic virus or derived from malignant tissue, since reducing the incubation period or the probability of commitment can convert a population with limited growth potential to one which grows indefinitely.

481

and so becomes the most stable conformation. It is also possible that in the extended form it will be easier for the  $N^+$  group of one molecule to approach close to the ring system of another molecule and polarize it, so regaining the polarization energy. It is proposed to apply a recent model (Hylton, Christoffersen & Hall, 1974) of a non-polar solvent to investigate the most stable configuration for these molecules in solution.

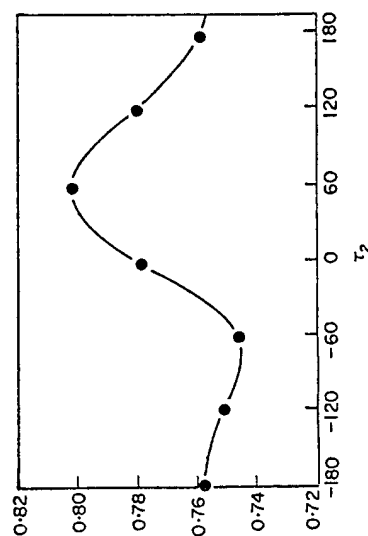


FIG. 4. Charge due to  $\pi$  electrons at carbon atom  $C_1$  of phenethylamine for  $\tau_1 = 90^\circ$ .

It is interesting to note that the electron density at the ortho position, which is the one most sensitive to the side chain configuration, is considered to be a factor in the receptor-substrate interaction of these molecules (Fujita & Ban, 1971).

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### 1. Introduction

It is well established that cultures of human diploid fibroblasts cannot be grown indefinitely in culture (for a review, see Hayflick, 1972). Primary cultures (phase I) give rise to populations of cells which divide for numerous cell generations (phase II), but which eventually lose their capacity for proliferation (phase III). Human fibroblasts which have been transformed by an oncogenic virus, such as SV40, or cells which have been derived from malignant tissue, such as the HeLa line, can be grown indefinitely in culture. These permanent lines are heteroploid and have morphological and growth characteristics which are quite distinct from diploid strains.

In a population of growing cells, there is continual selection for those with the shortest generation time. It would therefore be predicted that slow growing senescent cells would be eliminated, leaving the healthy ones to replenish the population. The problem therefore is not to explain the immortality of transformed lines, but rather to account for the inability of cellular selection to keep diploid cultures in a healthy growing condition. In this paper we develop a model which may help to surmount this problem. We suggest that senescence builds up very slowly in individual cell lineages, and show that an increase in its rate of development could convert a population of finite growth to one which grows indefinitely. A preliminary account of the model has been previously published (Holliday, 1975); here we present a full mathematical description.

### 2. The *In Vitro* Propagation and Ageing of Diploid Fibroblasts

Fibroblasts can only grow and divide when attached to glass or some other solid surface. When the surface area is covered with a monolayer of cells, cell division becomes very slow or ceases altogether, owing to the phenomenon widely referred to as contact inhibition. The confluent cells are detached from the surface with trypsin and the resulting suspension inoculated into containers with fresh medium. They then reattach to the surface and, after a lag of about a day, begin to divide. If a population from one container is inoculated to two of the same size, growth to confluence produces one population doubling (a 1:2 split subculture, or one passage). Individual laboratory populations normally consist of  $10^5$ – $10^7$  cells. The lifespan of a culture is known to be largely dependent on the number of population doublings or passages, rather than chronological time (Hayflick, 1965). The foetal lung strains WI-38 and MRC-5, which have been widely used in experimental studies, achieve about 50 and 60 population doublings respectively (Hayflick, 1965; Thompson and Holliday, 1973).

Fibroblast cultures from adult skin average 35 population doublings (Martin, Sprague & Epstein, 1970).

It is essential to realize that the number of population doublings cannot be equated with the number of cell divisions which occur before death. As non-viable cells begin to accumulate, the viable ones must undergo more than one division if the population as a whole is to double in number. The disparity between cell generations and passages becomes very pronounced towards the end of the lifespan (see Good, 1972). In addition, the number of divisions prior to and during phase I is obscure. The tissue from which the cells grow already contains many fibroblasts, but it is not possible to determine how many actually proliferate to produce the population in the primary culture. Thus, as well as the observed population doubling *in vitro*, there is an unknown number of cell divisions *in vivo* and in phase I, together with the additional cell generations which occur during the passing of senescent cultures. The total lifespan of fibroblasts may easily be over 150 cell generations.

Transformed heteroploid cell lines are propagated in the same way as diploids, but confluent cultures continue to divide. If the culture is not split, the cells may pile up in multiple layers, or become detached from the surface. There seem to be no well documented studies on the viabilities of transformed cells. Since non-disjunction of chromosomes leads to continual variability in chromosome number, it is obvious that non-viable cells lacking one complete haploid genome will be quite frequent, but whether individual cells die from other causes is unknown.

### 3. Commitment to Senescence

We can be certain that phase III populations contain only senescent cells, since it is impossible to select healthy long lived subclones from them. As we have mentioned, if the first events in senescence lead to an immediate reduction in the rate of cell division, then these cells would be selected against and the population would remain in a healthy condition indefinitely. Therefore, whatever the cause of ageing, we can conclude that cells must be committed or irreversibly destined to become senescent sometime before any visible signs of degeneration and reduction of the growth rate are actually observed.

Support for this general conclusion comes from detailed experimental studies in a fungus, *Podospora anserina*, which also has a finite lifespan in culture (Rizet, 1953; Marcou, 1961; Smith & Rubenstein, 1973). This organism grows at a constant rate for many days, weeks or months (the

longevity depending on the strain and the environmental conditions), but invariably this proliferation is followed by degeneration and death. The senescent condition is known to be determined by the cytoplasm not the nucleus. In studies of culture pedigrees, a clone is grown and then subcultured to two, then four, then eight tubes of agar medium, and so on. It is observed that a subset of cultures, for instance eight or 16 derived from an initial tube, all become senescent at the same time, whereas other equivalent sets of the same age may still be growing. However, when these in turn die, they always do so in subsets of the same size. From these and many other observations Marcou (1961) could show: (1) that the determinant for senescence arose at random in individual cultures; (2) that after the determinant appeared, the growth rate remained constant for many subcultures, and (3) that the time from the appearance of the determinant to senescence, the incubation period, was constant for any one strain kept in a uniform environment. It was surprising that this period was very long, at least half the median lifespan of a set of parallel cultures. These results have been confirmed and further documented by Smith & Rubenstein (1973).

The model we propose to explain the difference in growth potential in diploid and transformed cells is derived from these observations, but we apply it to lineages of cells rather than to whole cultures. We propose that commitment to senescence depends on cell division of fibroblasts, and that there is a given probability for each daughter cell that it will become committed. Committed cells are assumed never to revert to the uncommitted state. After commitment, the growth rate remains constant for many cell generations, but at the end of the incubation period the whole subclone derived from the committed cell dies out.

We shall not discuss molecular or cellular mechanisms of ageing here (for a recent review see Orgel, 1973), but merely mention three possible types of event which could be consistent with our general model.

(1) Orgel (1963) has pointed out that errors in protein synthesis could lead to an irreversible and exponentially growing error catastrophe, in which the accuracy of protein synthesis finally breaks down completely. Alternatively, a steady state level of errors might occur (Orgel, 1970). Uncommitted cells may have such a steady state, but this could be a metastable condition with a given probability of changing into the irreversible state. A particular critical error, or the coincidence of several errors, would produce a committed cell; the incubation period is then the time it takes to develop a lethal error catastrophe.

(2) Uncommitted cells can be thought of as an immortal stem line, from which committed cells are derived by cell division. In these cells a programme or clock is set which allows the cell to divide only a specified number of

times. Mechanisms for clocks which can count cell divisions have recently been proposed (Holliday, 1975; Holliday & Pugh, 1975).

(3) Commitment could be due to an event in a cytoplasmic particle which replicates independently of the nucleus. For instance, a defective mitochondrion may replicate a little faster than the normal organelle (Carnevali, Morpurgo & Tece, 1969). When, after many divisions, the normal functional mitochondria are diluted out, the cells become non-viable.

#### 4. The Structure of the Model

Cells can be divided into three classes: (1) uncommitted cells; (2) committed cells, and (3) dead cells. Committed cells can be further sub-divided into classes characterized by the number of elapsed cell divisions since commitment. Any difference between dead cells and those unable to divide is immaterial to a discussion of population growth. We assume that all such cells are passively transmitted, rather than lost, during routine subculture. (This assumption is reasonable, since it is known that many chemical or physical agents which kill cells do not prevent their attachment to solid surfaces, but is not essential to the general conclusions we reach. This point is discussed in more detail later).

A primary population of uncommitted cells of size  $N$  cells is split 1:2, and each subculture is allowed to grow until the population has doubled. Each subculture is then split 1:2 and so on until it is observed that no further growth occurs or until it is concluded that the population is immortal. Clearly the total number of subcultures increases rapidly and soon becomes prohibitively large for routine laboratory handling. It is necessary to discard all but a few subcultures, these being assumed to be representative of the total population.

During division of an uncommitted cell we assume a probability  $P$  for each daughter cell that it becomes committed, and a probability  $1-P$  that it remains uncommitted. We thus have three possible outcomes to the division of an uncommitted cell (see Fig. 1).

Commitment is irreversible, so that division of a committed cell always produces two committed cells. We assume that the path from first

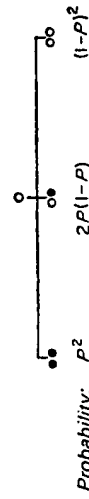


FIG. 1. Three possible outcomes to the division of an uncommitted cell. ○, uncommitted; ●, committed.

commitment is the same for any cell, and we define the incubation period  $M$  as the number of cell divisions which elapse between commitment and death.

The committed cells can then be divided into  $M$  distinct subclasses, the first of which contains newly committed cells, the second of which contains cells that have divided exactly once since commitment, and so on. The last subclass contains cells that have divided exactly  $M-1$  times since commitment and that will die after their next division.

The total population thus consists of  $M+2$  classes, one of uncommitted cells,  $M$  of committed cells and one of dead cells. We label these classes  $U, C_0, C_1, C_2, \dots, C_{M-1}, D$ . The subscript for the committed classes denotes the number of elapsed divisions since commitment. The population structure is determined by the numbers of cells of each class and is conveniently represented by an  $M+2$  dimensional column vector whose  $i$ th element denotes the number of cells in the  $i$ th class,  $U$  being the first class and  $D$  the  $(M+2)$ th.

Transitions from one class to another occur at each cell division. The transitions from  $C_0 \rightarrow C_1, C_1 \rightarrow C_2, \dots, C_{M-1} \rightarrow D$  are determined with certainty, i.e. a single cell in class  $C_n$  divides to produce two cells in class  $C_{n+1}$ , and similarly for transitions  $C_{M-1} \rightarrow D$ . Cells in class  $D$  remain there without division. Transitions  $U \rightarrow C_0$  occur by chance as described above. It is usual, however, for the number of cells under consideration to be large ( $N = 10^5-10^7$ ), and it is reasonable to neglect random fluctuations in the numbers of transitions  $U \rightarrow C_0$ , at least initially. Thus each cell in class  $U$  produces, on average,  $2P$  cells in class  $C_0$  and  $2(1-P)$  cells in class  $U$  at each division.

A natural discreteness is imposed on the time scale of the process by the assumption that transitions between classes occur only during cell divisions. We assume for the present that all live cells divide at the same rate, and we define the unit of time for the process as the interval between subsequent divisions. (The assumption that cells do not divide more slowly as they approach death is probably unjustified, but we make it to simplify the mathematical treatment. Slowing down the growth rate towards the end of the incubation period will not significantly alter the conclusions we reach.)

Let  $\mathbf{x}'$  denote the column vector describing the population at time  $t$ , i.e. after  $t$  divisions. We define a "transition matrix",  $\mathbf{T}$ , such that  $\mathbf{T}$  is the  $(M+2) \times (M+2)$  matrix whose  $i, j$ th element is the average number of cells produced in class  $i$  by division of a single cell in class  $j$ . Then

$$\mathbf{x}^{t+1} = \mathbf{T}\mathbf{x}'.$$

For a given initial population  $\mathbf{x}^0$  equation (1) defines the population process.

According to the assumptions made,  $\mathbf{T}$  takes the form:

$$\mathbf{T} = \begin{pmatrix} 2(1-P) & 0 & 0 & 0 & \dots & 0 & 0 & 0 \\ 2P & 0 & 0 & 0 & \dots & 0 & 0 & 0 \\ 0 & 2 & 0 & 0 & \dots & 0 & 0 & 0 \\ 0 & 0 & 2 & 0 & \dots & 0 & 0 & 0 \\ \vdots & \vdots & \vdots & \vdots & \ddots & \vdots & \vdots & \vdots \\ 0 & 0 & 0 & 0 & \dots & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & \dots & 2 & 0 & 0 \\ 0 & 0 & 0 & 0 & \dots & 0 & 2 & 1 \end{pmatrix}$$

### 5. Limiting Behaviour of Theoretical Population as $t \rightarrow \infty$

It is intuitively clear, from the irreversible nature of the ageing mechanism, that the population must eventually either become extinct or assume a stable distribution over the classes with a steady overall growth rate.

By definition, the limiting class distribution remains unchanged from one time unit to the next, although the numbers of cells in each class may be increasing. If we denote the limiting class distribution of the population by  $\mathbf{x}^\infty$  (unique but for a scalar multiplier), then we must have  $\mathbf{T}\mathbf{x}^\infty = \lambda\mathbf{x}^\infty$  for some constant  $\lambda$ , the limiting overall growth rate.

The possible values for  $\lambda$  are the eigenvalues of  $\mathbf{T}$ , and the corresponding vectors  $\mathbf{x}^\infty$  are the respective eigenvectors (see, for example, Kahan, 1969). For a real population we require  $\lambda \geq 1$ , as  $\lambda < 1$  means that the population size is decreasing, an impossible situation when dead cells remain in the population.

The only non-zero eigenvalues of  $\mathbf{T}$  are 1 and  $2(1-P)$ . If  $P \geq 0.5$ , then  $2(1-P) \leq 1$  and we have only one possible value for  $\lambda$ , i.e. 1, which means that the population must eventually become extinct. If  $P < 0.5$  then, provided the initial population contains some uncommitted cells, the number of uncommitted cells after  $t$  divisions is

$$\mathbf{x}_1^t = [2(1-P)]^t \cdot \mathbf{x}_1^0 > \mathbf{x}_1^0 > 0$$

for all  $t > 0$ , so that extinction ( $\lambda = 1$ ) is impossible and we must eventually have a stable population structure with a steady overall growth rate of  $\lambda = 2(1-P)$ . In the trivial case when the initial population contains no uncommitted cells, the population is always mortal with a lifespan less than  $M$  cell divisions.

T.B.

### 6. Short Term Development of the Theoretical Population

The previous section describes how the theoretical cell population may be expected to behave over a long period of time. It is unlikely in practice that the assumptions of the model conform sufficiently closely to reality to allow more than approximate long term predictions, and we now examine more closely the short term development of a population consisting initially of uncommitted cells only.

This can be divided into two stages:

*Stage 1.* During the first  $M$  (incubation period) cell divisions the population doubles with each cell division. No dead cells are present.

*Stage 2.* At the  $(M+1)$ th and subsequent divisions some cell deaths will occur. The population then consists of a mixture of live and dead cells and no longer doubles with each division. According to whether  $P$  is greater or less than 0.5, the population rapidly becomes extinct or approaches a stable distribution.

We are assuming that it is not possible to distinguish visibly between cells of different classes, and so one is dependent upon observations of the rate of population growth for information about the population structure. Three parameters, initial population size  $N$ , probability of commitment  $P$  and incubation period  $M$  completely determine the model, and we are therefore interested in the effects on population growth of varying one or more of these. The initial population size  $N$  will usually be known, and we shall concentrate attention on  $M$  and  $P$ .

The value of  $M$  determines the time of transition from Stage 1 to Stage 2, i.e. the time at which we should detect a change in the rate of growth of the population.

At time  $M-r$  ( $r = 0, \dots, M$ ) the number of live cells is

$$\sum_{i=1}^{M+1} x_i^{M-r} = 2^{M-r} \cdot N$$

while the number of dead cells is

$$x_{M+2}^{M-r} = 0.$$

At time  $M+s$  ( $s \geq 0$ ) the number of live cells is

$$\sum_{i=1}^{M+1} x_i^{M+s} = 2^{M+s}(1-P)^s \cdot N$$

while the number of dead cells is

$$x_{M+2}^{M+s} = 2^{M+1} N \cdot P \sum_{j=0}^{s-1} [2(1-P)]^j.$$

We find that subsequent to the occurrence of the first deaths, i.e. after time  $M$ , the proportion of live cells in the population is always independent of  $M$ . Thus the change in the growth rate is determined solely by the value of  $P$ .

Figure 2 shows the development of the population, as determined by computer simulation, for a range of values of  $P$ . For  $P > 0.6$ , the population becomes rapidly extinct, not achieving any further doublings. For  $0.5 \leq P < 0.6$ , a few further doublings occur before extinction. For  $P < 0.5$ , the population adopts a reduced but steady rate of growth and becomes immortal. In all cases the transition from Stage 1 to Stage 2 occurs rapidly.

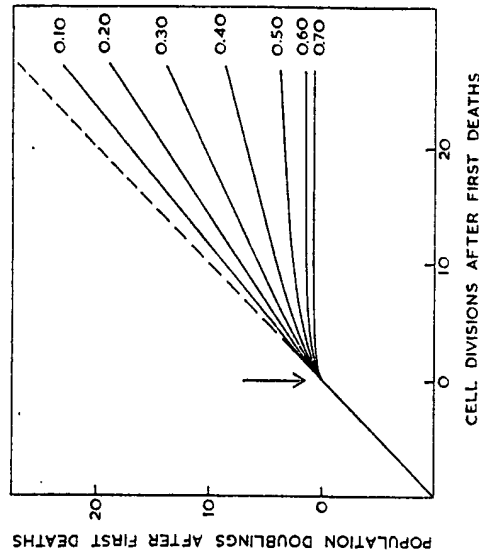


FIG. 2. Predicted transitions from Stage 1 to Stage 2 for a range of values of  $P$ , the probability of commitment. The change in growth rate depends only on the value of  $P$  and begins when the first cell deaths occur. The curves do not represent the full lifespan of the cultures.

### 7. Dilution of Uncommitted Cells

The description so far relates to the whole cell population, and it is important at this point to recall the culturing routine. The initial population of size  $N$  is split 1:2, each subculture is allowed to double in size, and is then again split 1:2. This process is continued with the majority of subcultures in fact being discarded at each stage.

While, in theory, this subdivision of the population should have no effect on the overall growth of the virtual population, in practice it is important to recognize that at any time no single unit of the population contains more than  $N$  cells. The accumulation of committed cells dilutes the uncommitted

cells, although the total population of uncommitted cells may be increasing. There may come a point where, in a culture of strictly limited size, the uncommitted cells are so dilute that there is a large probability of obtaining a subculture with no uncommitted cells, which is thus certain to become extinct.

Both the initial and continuing population size  $N$  and the incubation period  $M$  now play an important part in determining the mortality or immortality of the population. If  $N$  is too small or  $M$  too large, the uncommitted cells may be diluted out before any deaths occur and then, whatever the value of  $P$ , the population is mortal.

In any real population, the point at which the last uncommitted cells are lost is determined by chance, but will usually occur when the total number of uncommitted cells in any subculture is very small. If we assume, as an approximation, that this occurs when there is just one uncommitted cell in  $N$ , then for given  $N$  and  $P$  we can determine the maximum possible value for  $M$  such that the uncommitted cells do not become diluted out. Figure 3 shows, for two values of  $N$ , which values of  $P$  and  $M$  give rise to immortal populations. At  $P = 0.5$  the curves dividing the sets of parameter values into those for mortal and immortal populations are discontinuous. This is

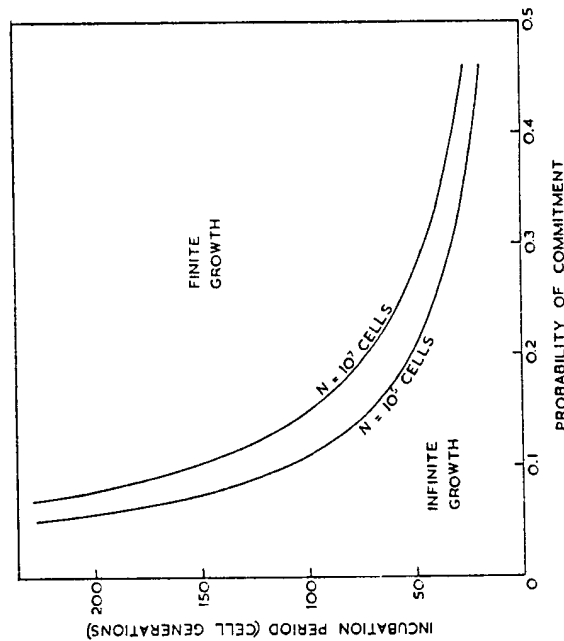


Fig. 3. For two population sizes, the curves divide the possible values of  $M$ , the incubation period, and  $P$ , the probability of commitment, into two sets, one producing an immortal population (i.e. unlimited growth) and the other producing a mortal population (i.e. finite growth). For  $P > 0.5$  (not illustrated) the population is always mortal. At  $P = 0.5$  a change in cause of mortality (see text) produces a discontinuity in the curves.

due to a change in the cause of mortality, as already discussed. We illustrate these curves only for  $P < 0.5$ ; the population is always mortal if  $P > 0.5$ .

It is perhaps surprising that a short incubation period with a resulting early death for individual committed cells increases the chances of immortality for the population as a whole (provided of course that  $P < 0.5$ ). Careful consideration of the process shows, however, that, if committed cells die quickly, the transition from Stage 1 to Stage 2 occurs while a reasonable proportion of the live cells are still uncommitted. In Stage 2 more than one cell division is required to achieve a population doubling, and, provided  $P < 0.5$ , each uncommitted cell produces, on average, two uncommitted cells between successive population doublings so that the proportion of uncommitted cells in the population remains constant.

If we consider the behaviour of a population in which the uncommitted cells have become diluted out before the transition to Stage 2, we find that there is initially little change in the growth of the population, since the uncommitted cells would, if present, have been too few to make any noticeable contribution to the growth rate. The population continues to double in size with each cell division (Stage 1), until the first deaths occur when, as described, it changes to a reduced but steady rate of growth (Stage 2). This stage continues as the committed cells steadily approach lethality. When the last of the committed cells approaches death, the rate of population growth begins again to diminish and a final stage of development (Stage 3), consisting of a steady "tailing off" to extinction, takes place. Figure 4

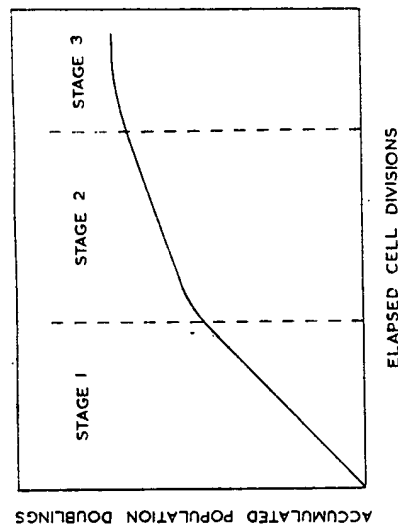


Fig. 4. Illustration of the three stages of population growth. In Stage 1 all cells are live and the population doubles with each cell division. In Stage 2 dead cells are present and the live cells therefore have to divide more than once to achieve a population doubling. The last uncommitted cells are lost by dilution (see text) before the end of Stage 1, so that Stage 3 occurs as the most recently committed cells approach death.

illustrates the three stages of population growth. It is essential not to confuse our Stages 1, 2 and 3 with Hayflick's phases I, II and III of fibroblast growth.

We have assumed throughout the development of the model that dead cells are transmitted rather than lost during subculture. This assumption is not likely to hold exactly, and we briefly consider the alternative extreme that all dead cells are lost. It is easily shown that in this case the population behaviour is very little altered. The transition from Stage 1 to Stage 2 growth would occur one cell division earlier and would be rather sharper. The rate of population doubling in Stage 2 would be the same, so the change of slope would be unaltered. The criterion determining whether the uncommitted cells are lost by dilution is completely unaffected. The rate of doubling in Stage 3 is slightly reduced, and the overall lifespan is a little shorter.

### 8. Experimental Observations

Our model is based on certain simplifying assumptions, in particular that the incubation period,  $M$ , is constant for all cell lineages, and that the interdivisional times are the same throughout the lifespan. The general features of the model would still be valid if  $M$  varied about a mean value, as we might expect would be the case in real populations, and if the interdivisional time increased towards the end of the lifespan. (The mathematical treatment would then become very complex, and existing experimental data is in any case insufficient to justify further elaboration of the model.) The upshot would be that Stages 1, 2 and 3 would tend to merge into each other, instead of giving the sharp transitions shown in Fig. 4. In view of this, it is surprising that a growth experiment carried out some years ago by one of us (R.H.) with MRC-5 fibroblasts gives a close fit to the model's theoretical prediction. The population in question was merely a control in an experiment in which the effect of 5-fluorouracil on *in vitro* ageing was under examination (see Holliday & Tarrant, 1972). Starting at passage 22 the cells were split 1:4 as rapidly as possible, i.e. as soon as they approached confluence, and at each subculture the cell population was counted. The confluent population averaged about  $10^6$  cells. The cumulative population doublings are plotted against time and passage number in Fig. 5. For 20 doublings the growth rate was constant, but at passage 42 the rate suddenly drops by 45% and then remains constant for about 13 doublings. Subsequently the culture began to phase out. From the change in the rate of growth at passage 42-44, we calculate that  $P = 0.275$ .  $M$  cannot be less than 42, and we predict that the population should die out at passage 66, as was observed. The prediction is based on the assumption that the last committed cells are lost when their frequency is  $10^{-5}$ . The theoretical curve is given in Fig. 5. The fact that the

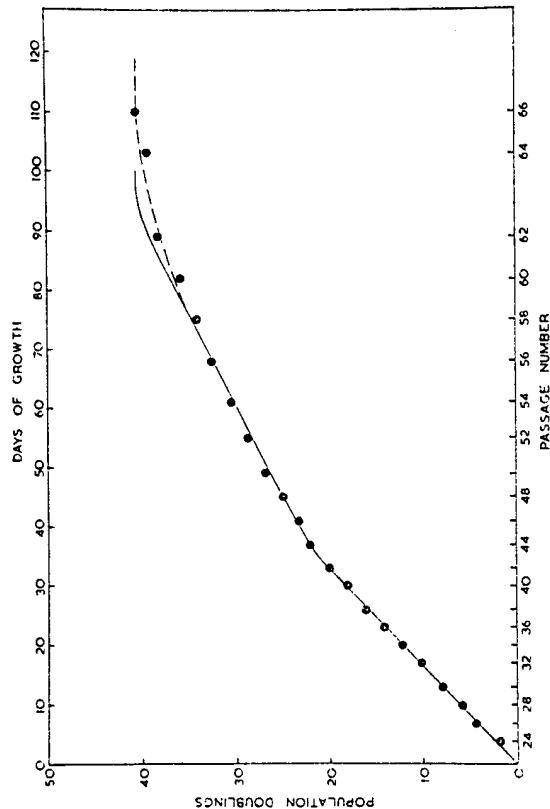


Fig. 5. Comparison of observed and predicted population growth. MRC-5 fibroblasts were split 1:4 as they approached confluence, and at each subculture the cells were counted with a haemocytometer. (For media and methods, see Holliday and Tarrant, 1972.) The solid curve shows the predicted growth of the population for  $P = 0.275$  and  $M = 43$ , assuming that the last uncommitted cells are lost when their frequency is  $10^{-5}$ . The displacement of the observed growth curve (dashed line) to the right of the predicted would be expected if the cells divide more slowly at the end of their lifespan.

later experimental points are to the right of the theoretical curve indicates that the rate of cell division slows down in phase III.

Published observations on fibroblast populations are in general rather than in specific agreement with our model. For instance, Cristofalo & Sharf (1973) found that the population of non-viable WI-38 cells (as determined by their inability to incorporate [ $^3\text{H}$ ]-thymidine into DNA) increased slowly during phase II and then much more rapidly in phase III. We would predict fully viable cells in Stage 1, a constant proportion of inviable cells in Stage 2 and an increasing proportion in Stage 3.

Smith & Hayflick (1974) sampled individual WI-38 cells at different passage levels and measured the size of the clones derived from them. They found that populations as early as passage 8 contain a substantial proportion (about 30%) of cells which had very limited growth potential (less than ten divisions). Cells from later passage levels had a higher proportion of such cells. In Stage 2 we would predict a similar heterogeneity, that is some cells would be dead, some capable of one division, some two, some three and so on, and the proportion capable of prolonged growth would certainly

decrease at later passages. Although from Fig. 5 we would not have expected that passage 8 cells would have reached Stage 2, it is difficult to compare data from the different strains when we are completely ignorant about their prior history. It is impossible to know whether MRC-5 and WI-38 of the same passage number have undergone approximately the same number of cell divisions. The greater growth rate and longer lifespan of MRC-5 suggests that the primary culture of these cells was more juvenile, and observations by R. Cox (unpublished data) indicate that a very high proportion of early passage cells can form large clones.

Our model makes a specific prediction which is at present under experimental test. The longevity of diploid fibroblasts should be a function of population size, but we would expect to see very little difference in lifespan between populations of, say,  $10^5$  and  $10^7$  cells. However, if the population is drastically reduced in size at the appropriate time, then a reduction in lifespan should be seen in a large proportion of cases. A bottle-neck experiment of this type should give the following results. At early passages, when the proportion of uncommitted cells is high, a bottle-neck will have no effect on longevity. Later on a bottle-neck will make the dilution out of these cells more likely, and the subsequent population (which has been allowed to grow to the normal size) will often have a reduced lifespan. Finally, once the uncommitted cells have in any case been lost, a bottle-neck will again have no effect on final lifespan. Should these specific predictions be borne out, it would be very hard to devise an alternative explanation to the one we have put forward.

## 9. Conclusions

Our model for *in vitro* ageing depends on three parameters: the probability of commitment  $P$ , the incubation period  $M$  and the population size  $N$ . For finite populations,  $M$  cannot be greater than the total lifespan. If we assume the latter is 100 population doublings, then for populations of normal laboratory size,  $P$  cannot be less than 0.1. If  $M$  was half this lifespan, then  $P$  could be 0.20–0.28, depending on the actual value of  $N$ . The results in Fig. 5 indicate that  $P$  is 0.275 and  $M$  is  $\geq 43$ . This incubation period seems extremely long, but we should recall that in *Podospora* the experimentally determined incubation period can be at least half the average lifespan.

Since the value of  $N$  is essentially the same for diploid and transformed cells, to change a population of finite growth to one which is permanent we must either postulate a reduction in  $P$ , or in  $M$ . Earlier we suggested three possible theories of cellular ageing which might be compatible with our model. The "mis-replicating organelle" hypothesis is very unlikely to be

true for mitochondria, since respiratory function is normal in Phase III cells (Hakami & Pious, 1968; Cristofalo, 1970). The second possibility, that fibroblast ageing is based on a programme or clock, is hard to reconcile with experimental data (Holliday, 1975). On the other hand, the protein error theory, although far from established, is at least supported by some experimental evidence (Holliday, 1969; Lewis & Holliday, 1970; Holliday & Tarrant, 1972; Lewis & Tarrant, 1972). We have no means of knowing the rate at which errors might increase. Our model requires a slow build up of errors: 40 divisions, or 30 days for cells dividing continuously with an 18 hr generation time. There is evidence that a lethal build up of errors in the *leu 5* strain of *Neurospora* takes 3 days (Lewis & Holliday, 1970), and the rate of protein synthesis or the doubling time for *Neurospora* is about ten times that of fibroblasts.

If ageing is due to the accumulation of errors in macromolecules, it seems improbable that transformed cells would be less likely to initiate an error catastrophe, i.e. have a lower  $P$  value. However, once it has been initiated, it might well develop more quickly in these cells than in diploid ones. For instance, transformed cells may have less ability to degrade abnormal proteins. It is outside the scope of this article to discuss the actual process of transformation, but if it occurs in Stage 2 or Stage 3 cells it would provide an exception to our assumption that committed cells never give rise to uncommitted ones. [Reversal of the ageing process in committed cells by stringent environmental treatments, such as starvation or low temperature, is well documented in *Podospora* (Marcou, 1961).]

At first sight it is paradoxical to conclude that a more rapid build up of errors in a cell lineage will allow continuous growth of the whole population. However, as we mentioned at the outset, the problem is not so much to explain the immortality of a heterogeneous transformed population, since cellular selection will always eliminate sick cells, but rather to understand why cellular selection does not also permit diploid populations to grow indefinitely. Our model attempts to provide a solution.

Other surprising conclusions can be drawn from our model. One of the standard tests of the protein error theory is to artificially increase the error frequency with amino acid or RNA base analogue treatments, and then determine whether the lifespan is shortened. Although it is true that inducing irreversible error catastrophes may shorten the lifespan of populations of cells, reducing the time it takes for errors to reach the final catastrophe could have the reverse effect. Analogues might therefore either reduce or increase lifespans, depending on which effect was the greater. Finally, if our model has validity, we can conclude that diploid fibroblasts may in fact be immortal, contrary to the numerous experimental observations which



LETTER TO THE EDITOR

# Thermodynamic Considerations of the Deoxyribonucleic Acid Helix-Cruciform Transition

It has been shown that denatured eukaryotic DNA can fold back on itself to form hairpin (cruciform) structures (Wilson & Thomas, 1974). To form cruciforms, these DNA regions must contain inverted base sequence repetitions (called palindromes; Wilson & Thomas, 1974) which read the same in both directions (e.g., if a palindromic sequence of one strand reads 5'ATGCGCAT3', the complementary strand must read 3'TACGCGTA5'). Palindromes have been reported for *Escherichia coli* DNA *lac* repressor site (Maniatis, Ptashne, Barrell & Donelson, 1974) and for a variety of RNAs (references cited in Wilson & Thomas, 1974). The double stranded helical DNA at a palindrome could separate and reform into a cruciform configuration (Fig. 1). This DNA configuration could act as recognition sites for protein mediated nucleic acid processes (recently reviewed by Sobell, 1973; Wilson & Thomas, 1974; Lewin, 1974).

For the helix-cruciform transition shown in Fig. 1, each structure (helix or cruciform) can only reach the other by passing through the coil state. The energy of the helix-coil transition is probably different from that of the

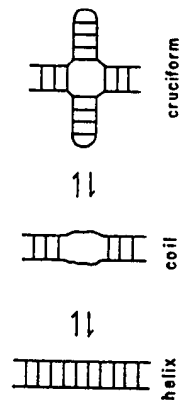


FIG. 1. Illustration of the possible DNA forms in the transition of a region containing a palindrome from a double helix to a cruciform.

cruciform-coil transition. If these energies are sufficient to prevent the DNA from attaining an equilibrium distribution between the helix and cruciform states, a metastable situation would arise in which the state of the DNA would not be a minimum free energy state. In this event, the helix-cruciform transition would be a sharp nonequilibrium process with hysteresis. For this system, hysteresis means that, if the DNA distribution in the helix and

have been published. We predict, however, that such immortality will never be seen in laboratory populations, but only in those of enormous size. If a way could be found to separate uncommitted cells from committed ones, then the population could be kept growing indefinitely.

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